Reference: *Biol. Bull.*, **144**: 462–470. (June, 1973)

EFFECTS OF SPERM CONCENTRATION, SPERM AGING, AND OTHER VARIABLES ON FERTILIZATION IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* L.

GEORGE GORDON BROWN AND JACK R. KNOUSE

Department of Zoology and Entomology, Iowa State University, Ames, Iowa 50010 and Duke University Marine Laboratory, Beaufort, North Carolina 28516

Although the horseshoe crab, *Limulus polyphemus* L. and related species have been extensively used in reference to embryology (Lockwood, 1870; Packard, 1885; Kingsley, 1892; Iwanoff, 1932; Roonwal, 1944; and Sekiguchi, 1970), only recently has this species been used for significant fertilization studies. Such studies have involved fine structure of the initial sperm-egg interactions (André, 1963; Shoger and Brown, 1970), immunological approaches to these interactions (Cooper and Brown, 1972; Mowbray and Brown, 1973), and stereoscan observations (Brown and Humphreys, 1971). In examining fertilization in *Limulus* one must be impressed by the large number of spermatozoa (10^5 to 10^6) which can simultaneously attach to the egg surface and still allow normal development to occur. Since this phenomenon can be readily demonstrated, the present study is a quantitative approach to examine the effect of sperm concentration on fertilization and development.

Although in some animal species (*e.g. Arbacia*) a single sperm can approach, attach, penetrate and fertilize an egg (Lillie, 1919; Cohn, 1918), in most species the normal number of spermatozoa attaching to or in the immediate vicinity of the egg to be fertilized is usually greater than one (*cf.* Cohen, 1971; Austin, 1969a). Also, as demonstrated in *Arbacia* and other species too many spermatozoa contacting the egg simultaneously can cause pathological polyspermy resulting in abnormal development (*cf.* Austin, 1969b). Thus, we might examine the roles of several spermatozoa during fertilization. As readily demonstrated in several species of crustacean decapods and in *Limulus* large numbers of spermatozoa normally attach to the egg surface (Binford, 1913; Brown, 1966; Hinsch, 1971; Shoger and Brown, 1970). In these species each sperm not only attaches but also undergoes an acrosome reaction, penetrating the egg envelope, but not necessarily the egg plasma membrane. Better known are the conditions in various species of mammals, where many reacted spermatozoa are found associated with the cumulus and corona cells and the zona pellucida (Overstreet, 1970; Bedford, 1970; Franklin, Barros, and Fussell, 1970; Menge, 1971). Thus, in conclusion, during fertilization the phenomenon of several or many spermatozoa contacting and penetrating the egg envelope is common in species of diverse phylogeny. In many of these species, the initial contact and close associations with the egg envelope can be referred to as polyspermic attachment.

Since in *Limulus* polyspermic attachment occurs and each sperm undergoes the acrosome reaction simultaneously, the ultimate question concerns the control of sperm penetration into the egg proper. This study therefore involves in *Limulus*

an initial approach to determine the apparent necessity of a high sperm concentration in facilitating fertilization and some elucidation of the mechanisms involved. Following are observations demonstrating the unique relationship of sperm concentrations and normal development.

Materials and Methods

Specimens of the horseshoe crab, *Limulus polyphemus* L., were collected in the initial stages of this study from breeding grounds on Shackleton Banks near Beaufort, North Carolina, during the summer of 1969. In later experiments animals were either obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, or from the Florida Marine Biological Specimen Company, Panama City, Florida. Specimens were kept in running sea water tables at the Duke University Marine Laboratory (summer, 1969) and in "Instant Ocean" Culture Aquaria (15° C) at Iowa State University. Including all experiments approximately 50 male and 25 female horseshoe crabs were used.

In performing the studies described in this paper, a standard experimental procedure for fertilizing *Limulus* eggs was only established after numerous trials and errors. Following is a description of this standard procedure which quite obviously includes some variables referred to in the next section. Gametes were usually obtained by electrical stimulation after procedures previously described (Shrank, Schechtman, Shoger, and Bishop, 1967). The semen was obtained "dry," routinely diluted in sea water to a 10% sperm concentration, and stored at 4 to 5° C for 72 hours. Before using in experiments, the spermatozoa were checked for viability by placing one drop on a microscope slide containing egg sections (Mowbray and Brown, 1973). In most cases, except where variables were being tested, 1 ml of a 10% sperm concentration was mixed with approximately 100 eggs in a plastic centrifuge tube (the eggs adhere to glass). The eggs in all cases were collected in oviductual fluid ("dry") and immediately mixed with the appropriately prepared sperm concentration. These sperm-egg suspensions were allowed to sit with an occasional swirling for approximately 5 to 10 minutes or 25 to 30 minutes, washed several times, placed in parafilm-lined syracuse dishes with streptomycin-penicillin sea water (Perkins and Menzel, 1964) and observed for development. The per cent of development was based on the number of inseminated eggs to develop to the limb bud stage or into swimming larvae. Unfortunately, it usually took 8 to 10 days (room temperature, 22–25° C) before definitive development was observed. The variables tested in methodology were: (1) different types of egg collecting, (2) effects of seminal fluid, and (3) duration of sperm-egg mixing. After standards were obtained in these methods, experimentation involved the effects of different sperm concentrations and aging of spermatozoa on development.

Observations

Methods of collecting eggs

To observe if the per cent of development was related to the method of collecting, eggs were collected by three different methods: electrical stimulation, dis-

section, and oviduct caressing. The electrical stimulation method was referred to in the previous section. The dissection method involved killing the animal by exsanguination followed by immersion in warm water, dissecting away the dorsal region of the prosoma, and removing eggs directly from the long oviducts. The oviduct caressing method consisted of pressing the oviduct externally with the index finger. By moving the finger ventrally towards the genital opening, eggs could be forced out. In all cases, the collected eggs were immediately mixed with spermatozoa. In electrical stimulation 77.6% development resulted, with dissection 72.5% and with caressing method 57.0%. Since electrical stimulation was the most convenient, it was used in all following experiments.

Effects of seminal fluid on development

In order to determine the effects of seminal fluid on development, 5 ml of freshly prepared 10% sperm concentration was washed thrice by mild centrifugation with 10 ml of sea water and stored for 72 hours at 5° C before mixing with eggs. For comparison, another 5 ml of a 10% sperm concentration was treated similarly except it was left unwashed. In comparing washed spermatozoa (free of seminal fluid) and unwashed spermatozoa, a high per cent of development was obtained in both cases, 70.0% and 64.1% respectively. Since the difference in development between both types of sperm concentrations was insignificant, unwashed sperm concentrations were used in all experiments.

Duration of sperm-egg mixing

In order to determine an optimal time for sperm-egg mixing, approximately 500 eggs were mixed with 5 ml of a 10% sperm concentration. Immediately after mixing and at the following intervals: 5, 15, 30, and 60 minutes, samples of approximately 100 inseminated eggs were removed and washed. In addition, after each time period the sperm-egg mixture was examined for sperm motility. As noted in Table I, the per cent of development for each sample was reasonably high with the exception of the 15 minute sample. As a result either 5 to 10 minutes or 25 to 30 minutes were used as a standard time of mixing. As was noted when observing sea water samples from each sperm-egg mixture, sperm motility generally involved more than 90% of the total number of spermatozoa. Although the greatest decrease of sperm motility occurred after 4 to 5 minutes, a few motile spermatozoa were observed 10 to 15 minutes after mixing.

TABLE I

Duration of sperm-egg mixing

Time of mixing (min)	Number of experiments performed	Number of different females used	Number of eggs	Number of eggs developing	Per cent development
1	3	2	242	224	92.6
5	3	2	319	206	64.6
15	3	2	309	128	41.4
30	3	2	278	195	70.2
60	3	2	264	197	74.6

TABLE II

Aging of spermatozoa

Hours of aging	Number of experiments performed	Number of different females used	Number of eggs	Number of eggs developing	Per cent development
0–1	3	2	331	143	43.2
24	3	2	393	203	51.7
48	3	2	383	262	68.4
72	3	2	501	418	83.5
96	3	2	371	282	76.1

Aging of spermatozoa

For determining the effects of aging on sperm viability, 5 ml of a 10% sperm concentration was stored at 4 to 5° C for 96 hours. At each of the following intervals: 0, 24, 48, 72, and 96 hours, 1 ml of this sperm concentration was removed and mixed with approximately 100 eggs and the percent of development determined. As noted in Table II, the older sperm concentrations (24 to 96 hours) gave higher development than freshly collected spermatozoa. There was no testing beyond 96 hours. Since 72 hours of aging gave the best result (83.5%), this period of aging was generally used for all 10% sperm concentrations before they were used in experimentation.

Sperm concentration and development

In testing the effects of sperm concentration on development, a 10% sperm concentration (stored for 72 hours at 4 to 5° C) was serially diluted so that 1 ml of each of the following sperm concentrations was obtained: 10%, 1.0%, 0.1%, 0.01%, 0.001%, and 0.0001%. In addition 1 ml of a 100% sperm concentration was used. Since previous studies with extensive sperm dilutions in sea urchins showed a rapid loss of fertility during aging (Cohn, 1918), each of these sperm concentrations was immediately mixed with eggs. This was only a precautionary step since recent experiments in our laboratory have demonstrated that the lower sperm concentrations maintain the same fertility level at least 1 hour, a time well within the confines of this experiment.

The effects of serially diluted sperm concentrations on development are tabulated in Table III. Although some development was observed with all per cents of sperm concentration used, the higher average per cents: 68.3, 66.3, and 76.8, occurred with 1%, 10%, and 100% sperm concentrations, respectively. A noticeable decrease in development occurred with 0.1% sperm concentration (33.3%) and became more obvious at 0.01% (17.6%) and 0.0001% (2%) sperm concentrations.

Because of the recent emphasis on a quantitative approach to fertilization (Cohen, 1971), the number of spermatozoa for each sperm concentration was determined with a hemocytometer. From this the approximate number of spermatozoa per egg for each sperm concentration was determined (Table III). Finally, in order to double check the viability of spermatozoa and to determine an

Table III

Sperm concentration and per cent of development

Sperm concentration in per cent	Number of experiments performed	Number of different females used	Approximate number of spermatozoa/ml*	Approximate number of spermatozoa/egg*	Calculated number of attaching spermatozoa/egg	Number of eggs	Number of eggs developing	Per cent development
100	2	2	10^{10}	10^{8}	—	150	115	76.7
10	11	10	10^{9}	10^{7}	626,000	925	613	66.3
1.0	11	10	10^{8}	10^{6}	118,000	972	664	68.3
0.1	11	10	10^{7}	10^{5}	23,000	936	312	33.3
0.01	10	9	10^{6}	10^{4}	7,400	830	146	17.6
0.001	7	6	10^{5}	10^{3}	750	570	44	7.7
0.0001	5	4	10^{4}	10^{2}	130	499	10	2.0

* In all experiments, approximately 100 eggs were mixed with 1 ml of each sperm concentration.

approximate number of spermatozoa actually attaching to each egg, egg sections were employed. The methods for using *Limulus* egg sections are described in a separate paper (Mowbray and Brown, 1973). Basically, they involve freezing of whole eggs, frozen sectioning at 10 to 12 μ, mixing of 0.1 ml of sperm concentration, and the counting of spermatozoa attaching to a known egg area. From these sperm counts the approximate number of spermatozoa attaching to the whole egg could be determined with each sperm concentration by multiplying the sperm counts per egg section area (0.0056 mm^2) by the surface area of the whole egg (9.42 mm^2). For example, with an 1.0% sperm concentration the sperm count was 70.3, thus allowing a calculated 118,000 spermatozoa attaching to the whole egg (Table III). For each sperm concentration, the ratio of the total number of spermatozoa to egg surface area was approximately the same, regardless of whether using whole eggs or egg sections.

Discussion

Although the emphasis in this study is placed on sperm concentration and sperm aging and their effects on fertilization, the usage of a new research animal, *Limulus polyphemus*, in fertilization studies is also demonstrated. In addition, these studies with this species gametes are particularly unique because of *Limulus* phylogenetic relationship and the study of arthropod fertilization, which is unquestionably difficult due primarily to the evolutionary adaptation of this group to internal fertilization. In reference to the methods for this study the following variables are discussed: methods of collecting eggs, effects of seminal fluids, and duration of sperm-egg interactions.

As demonstrated in this study, fertilization and larval development are readily performed in the laboratory. The availability of *Limulus* and the ease in obtaining an abundance of viable gametes year around certainly enhances the usefulness of this species in fertilization studies. In reference to obtaining gametes, the electrical stimulation method has proven quite satisfactory, although unfertilized eggs, unfortunately, frequently became activated shortly (usually 5 to 20 minutes) after collecting and appeared to be developing normally for as long as

four days before they deteriorated. Other methods of collecting did not significantly prevent this egg activation. The presence or absence of seminal particles or fluids does not effect fertilization, although they probably play a very important role during sperm storage and sperm inactivity in the male animal. An adequate duration for mixture of the gametes is 5 minutes (Table I). Interestingly, this time is directly related to the duration of sperm motility which is 4 to 5 minutes from initial mixing. This implies that the sperm functional time is quite short unless attachment to the egg occurs. One side effect of mixing times is the low per cent of development occurring when eggs are washed 10 to 15 minutes after the mixing of gametes. Presumably a disturbance of a sensitive sperm penetration event during this time causes a malfunctioning in fertilization.

The aging of a 10% sperm suspension at 4 to 5° C for three days increases the per cent of development (Table II). Although no adequate explanation presently exists for this phenomenon, it is interesting that an increase in fertilizability of stored semen has also been observed with bull semen (Salisburg and Hart, 1970) and with chicken semen (Lodge, Fechheimere, and Jaap, 1971), although the latter case was *in vivo*. In their study, Salisburg and Hart interpreted this improvement as the selective death of abnormal spermatozoa. Stored *Arbacia* semen did not produce this effect (Goldfarb, 1918).

In *Limulus* a high sperm concentration per egg definitely enhances a higher per cent of development. As shown in Table III a 1% sperm suspension (1,000,000 spermatozoa/egg) resulted in 68.3% development of eggs into swimming larvae. In comparison, a 0.01% sperm suspension (10,000 spermatozoa/egg) and a 0.0001% sperm suspension (100 spermatozoa/egg) resulted in 17.6% and 2% respectively, a rapid decrease in development even though many spermatozoa were present. Although not numerically the same, there is an interesting comparison between *Limulus* and rabbit fertilization. Overstreet (1970) has observed that a high number of capacitated spermatozoa (10,000+) injected into the oviducts of ovulated rabbits ensures a higher per cent (90% or better) of fertilization and as the sperm number is reduced to 500–1500, a considerably lower per cent (30–50%) of development takes place.

In *Limulus*, many spermatozoa (approximately 10^6) can actually attach to the egg surface and normal development ensues (Brown and Humphreys, 1971). After attachment each sperm undergoes the acrosome reaction and forms an acrosomal filament which penetrates the tough egg envelope (Shoger and Brown, 1970). Interestingly enough, polyspermic attachment (or similar association) has been observed to occur normally in other species, for example, decapods (Binford, 1913; Brown, 1966; Hinsch, 1970), insects (Huettner, 1927), and some mammals (rabbits, Bedford, 1970). Studies by Bedford (1970) and Overstreet (1970) show that many spermatozoa (several hundred) come into contact with the rabbit egg cumulus and many have actually been observed to penetrate into the perivitelline space surrounding each egg. Mammal spermatozoa in contact with the egg cumulus and zona pellucida release the enzymes, acrosomal hyalauronidase and acrosomal proteinases, which aid sperm penetration (Yanagimachi and Teichman, 1972). Supposedly, a greater chance of sperm penetration occurs with numerous spermatozoa releasing these enzymes (*cf.* Austin, 1969a). Since sperm penetration studies in *Limulus* are not complete, whether a similar role could exist

can only be speculated. However, the existence of an acrosomal proteinase has been determined (Bennett and Brown, in preparation). Perhaps, in *Limulus,* a lytic or chemical reaction could result from the large number of reacted spermatozoa, forming canals or causing chemical changes in the egg envelope thus facilitating the movement of one or a few sperm nuclei towards the egg plasma membrane. An alternative would be that the process of penetrating the egg envelope (approximately 45 microns thick) may be selective enough to allow only certain genetically determined spermatozoa or those on specific penetrable sites to pass through the envelope. Quite possibly there may be a combination of these processes. Unfortunately, at present, whether or not polyspermy penetration occurs in *Limulus* is unknown. Since this phenomenon is common in many arthropods (insects, Huettner, 1927; Piko, 1961), the possibility does exist.

In considering all the aspects suggested above and in a superficial answer to the pleas of Cohen (1971) one cannot help but to reflect on the evolutionary significance of a large number of spermatozoa attaching to an egg. This reflection is enhanced by the fact that other arthropods (insects, decapods) share this phenomena, although not to the same degree. Judging from this study, apparently the answer is not merely a large number of reacting spermatozoa chemically facilitating the penetration of one or several spermatozoa, since even a low sperm concentration can occasionally fertilize an egg. One must speculate on why so many spermatozoa attach to *Limulus* eggs whereas other species have evolved various ways (micropyles, sperm dilution, complex reproductive systems, *etc.*) to reduce the sperm number reaching the egg. Apparently in *Limulus* selection for sperm number reduction has taken place within the sperm envelope, so that the selective mechanism does not affect spermatozoa simultaneously binding to the egg surface, but operates within the egg envelope allowing only a minimal number of sperm nuclei to reach the egg plasma membrane. Thus, it is possible that this envelope selective mechanism is variable enough so that while normally large numbers of spermatozoa are necessary for fertilization, occasionally small numbers can also successfully fertilize the egg. This latter would account for the observed development occurring with very low sperm concentrations.

This work was initiated at Duke University Marine Laboratory with the aid of a Postdoctoral Fellowship funded by a grant (GB-7443) from the National Science Foundation to Duke University. Special thanks to Dr. John D. Costlow and Dr. C. G. Bookhout for use of the many facilities of the Laboratory. We also thank Mr. Thomas E. Hess for technical assistance.

Summary

The horseshoe crab, *Limulus polyphemus,* is an excellent species for fertilization studies since viable gametes can be easily obtained on a year around basis.

In reference to methodology for *Limulus* fertilization studies, the following prove to be adequate: collection of gametes by electrical stimulation, unwashed or washed spermatozoa (free of seminal fluid), and 5 to 10 minutes duration for sperm-egg mixing.

The aging of a 10% sperm concentration at 5° C for three or four days increases the per cent of embryonic development. This increase may be due to the death of abnormal spermatozoa.

Higher sperm concentrations definitely enhance higher per cent of development. With the dilution of sperm concentration, the per cent of development was drastically reduced even though several hundred spermatozoa per egg were present. The significance of the high number of spermatozoa is speculated as a necessity for (1) the chance penetration of a special site, (2) the release of sperm enzymes causing a chemical lysis in the egg envelope, or, more favorably, (3) a selection mechanism determined by the egg envelope for special genetical spermatozoa.

The number of spermatozoa per egg is calculated for each sperm concentration. In addition, a method for calculating the number of spermatozoa actually attaching to each egg is formulated.

Finally, some speculations on the evolution of such a system as found in *Limulus* are presented.

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